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RT-PCR

For RT-PCR, total RNA was isolated by the RNeasy midi kit (QIAGEN). PCR primers used in Fig. 1 A are as follows: Gab1 (forward: 5′-cagttacgacattccgccaa-3′, reverse: 5′-ggattggctcggtaaagctg-3′; position: 915–1405); Gab2 (forward: 5′-gaagagtactggcagtgtggat-3′, reverse: 5′-caaagcaggaagctttttggc-3′; position: 3′UTR); Gab3 (forward: 5′-acagcttcacccagtgctga-3′, reverse: 5′-gtccagggccaaataatcca-3′; position: 1156–1638); actin (forward: 5′-cgtgggccgccctaggcacca-3′, reverse: 5′-ttggccttagggttcaggggg-3′). PCR primers used in Fig. 2 A are as follows: Gab1 (forward: 5′-ctggctcctcacaagcac-3′; reverse: 5′-cagctcttcacccgagac-3′; position: 386–1332), Gab2 (forward: 5′-tctgatgacaactacgtgccc-3′; reverse: 5′-ttacagcttggcacccttgga-3′; position: 1309–2001). Gab3 and actin primers used are the same as those in Fig. 1 A. We used the following PCR conditions: first cycle at 95°C for 3 min, followed by seven cycles, with denaturation at 95°C for 30 s and extension at 72°C for 1 min. Within these seven cycles, the annealing temperature was reduced by 1°C per cycle from 62 to 56°C. 28 cycles were then performed (denaturation at 95°C for 30 s, annealing at 56°C for 45 s, and extension at 72°C for 1 min).

The luciferase/GFP reporter assays

The 3′-UTR and its adjacent 200 bp of coding sequence of mouse Gab2 and full-length mouse Gab3 cDNA were subcloned into the pCS2-luciferase vector (Yu et al., 2002) to create Gab2 and Gab3 reporters, respectively. A second Gab2 reporter, pCS2-GFP-Gab2-3′-UTR, was generated by replacement of the luciferase gene in the pCS2-luciferase-Gab2-3′-UTR construct with GFP. shRNA constructs were cotransfected with either reporter into P19 cells. 48 h after transfection, luciferase activity was measured and normalized by β-gal activity. GFP expression was monitored by fluorescence microscopy.

JNK and p38 in vitro kinase assay

P19 cells were treated with 10 ng/ml bFGF, 1 μM RA alone, or in combination for 0, 0.5, 1, 5, and 10 h. 200 μg of lysates was immunoprecipitated by anti-JNK or anti–phospho-p38 antibody. Immune complexes were washed twice in 1×LB, twice in LiCl wash buffer (0.5 M LiCl and 0.1 M Tris-Cl, pH 8.0), and once with JNK kinase buffer (20 mM Hepes, pH 7.4, 10 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM EGTA, 0.2 mM Na_3VO_4, 1 mM DTT, and 0.03% Brij 35) or p38 kinase buffer (according to the manufacturer's protocol) and resuspended in 25 μl of kinase buffer containing 5 μCi of γ-[^{32}P]ATP (ICN) and 1 μg of the corresponding substrate, GST-c-Jun, or ATF-2. Incubation was for 30 min at 30°C. Kinase activity was quantified by a PhosphorImager (Bio-Rad Laboratories).

References

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